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Modeling of a spray drying method to produce ciprofloxacin nanocrystals inside the liposomes utilizing a response surface methodology: Box-Behnken experimental design

--Manuscript Draft--

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Abstract:	Spray drying was previously used to modify the physical form of the encapsulated ciprofloxacin drug to produce ciprofloxacin nanocrystals inside the liposomes (CNL). The purpose of the present study was to optimize CNL powder production by evaluating the response surface via design of experiments (DoE). Using the Box–Behnken (BB) design, the study independent variables were the protectant type (sucrose, trehalose or lactose), protectant amount, drying temperature, and spray gas flow. Individual spray drying experiments were performed at various set points for each variable followed by characterization of the produced powders. Liposomal particle size, drug encapsulation efficiency (EE%), liposomal surface zeta potential, and nanocrystal dimensions were the design dependant variables. By applying the least square regression method on the experimental data, mathematical models were developed using the mathematical software package MATLAB R2018b. Model reliability and the significance of the model's factors were estimated using analysis of variance (ANOVA). The generated CNL powders showed spherical to elliptical liposomal vesicles with particle sizes ranging from 98 to 159 nm. The EE (%) ranged from 30 to 95% w/w while the zeta potential varied between -3.5 and -10.5 mV. The encapsulated ciprofloxacin nanocrystals were elongated cylindrical structures with an aspect ratio of 4.0 - 7.8. Coefficients of determination ($R^2 > 0.9$) revealed a good agreement between the predicted and experimental values for all responses except for the nanocrystal dimensions. Sucrose and lactose were superior to trehalose in protecting the liposomes during spray drying. The amount of sugar significantly affected the characteristics of the CNL powders (p -value < 0.05). In conclusion, the DoE approach using BB design has efficiently modelled the generation of CNL by spray drying. The optimum processing conditions which produced high drug encapsulation (90%) after formation of nanocrystals and a vesicle size of ~125 nm utilized 57% (w/w) sucrose, an 80°C inlet temperature, and an atomization rate of 742 L/hr.

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Professor Jürgen Siepmann
Editor-in-Chief, International Journal of Pharmaceutics

Dear Professor Siepmann

We would be grateful if you would consider publication of this manuscript entitled **'Modeling of a spray drying method to produce ciprofloxacin nanocrystals inside the liposomes utilizing a response surface methodology: Box-Behnken experimental design'** in International Journal of Pharmaceutics.

In this manuscript, we described the optimization of spray drying method for the production of ciprofloxacin nanocrystals inside liposomes (CNL) powder by implementing response surface via design of experiments (DoE). Box–Behnken (BB) design was used to study the relationship between the independent variables (protectant type, protectant amount, drying temperature, and spray gas flow) and the dependant variables (liposomal particle size, drug encapsulation efficiency (EE%), liposomal surface zeta potential, and nanocrystal dimensions). Mathematical and statistical software (MATLAB and Design-Expert) were applied to construct the equations and confirm the model reliability. In the present study, the significant factors were identified and their effect on the CNL characteristics were described. Besides, the optimal independant variables values were determined and validated for confirming the model reproducibility. The DoE approach using BB design has efficiently modelled the generation of CNL by spray drying. The optimum processing conditions which produced high drug encapsulation (90%) after formation of nanocrystals and a vesicle size of ~125 nm utilized 57% (w/w) sucrose, an 80 °C inlet temperature, and an atomization rate of 742 L/hr.

Thank you for handling our manuscript.

Yours sincerely,

Professor Hak-Kim Chan (Corresponding author)

COMMENTS TO AUTHOR

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Title: Modeling of a spray drying method to produce ciprofloxacin nanocrystals inside the liposomes utilizing a response surface methodology: Box-Behnken experimental design

Authors: Isra Khatib, Michael Y.T. Chow, Juanfang Ruan, David Cipolla, Hak-Kim Chan

We thank the reviewers for their valuable time and efforts in providing constructive comments. We have now addressed all the comments and implemented changes to the manuscript accordingly.

Colour Codes:

Reviewers' Comments: Black

Responses to the comments: Green

Corrections and Text insertions in the manuscript: Red

Reviewer #1:

- ***Comment 1:***

In the first paragraph of the introduction, the author discussed the pulmonary delivery of the antibiotics including non-tuberculous mycobacterium. In the 2.2. experimental design section, pulmonary delivery was also considered as one of the selection factors. However, there are no pulmonary drug experiments performed in this study. In order to prove the significance of this study, the aerosol performance should be evaluated at least for the optimized process as well as the water content.

We agree with the reviewer and have included the *in vitro* aerosol performance and also the water content of the optimized formulations. Under Section **2. Materials and methods,** the following sections were added**:**

2.9. Evaluation of optimized formulations for respiratory delivery and controlled drug release

2.9.1. Moisture content: Thermogravimetric analysis (TGA)

2.9.2. In vitro aerosol performance

Under Section **3. Results and Discussion**, the following sections were added:

3.6. *Evaluation of optimized formulations for respiratory delivery and controlled drug release*

3.6.1. Moisture Content

3.6.2. In vitro aerosol performance

Please refer to the revised manuscript for more details.

- ***Comment 2:***

In the second paragraph of the introduction "Although the widely used lyoprotectants for spray drying liposomes are disaccharides e.g. sucrose, trehalose, and lactose", the term lyoprotectants was used for excipients of spray drying. However, a quick google search showed people don't use the term "lyoprotectants" for excipients for spray drying.

Lyoprotectants are hydrophilic molecules incorporated into the formulation to overcome denaturation (in case of proteins) and preserve stability during lyophilization (Emami et al., 2018). They provide an amorphous glassy matrix, bind to the compound through hydrogen bonding, and replace the water molecules that are removed during the drying process. This helps to maintain the compound conformation, minimize degradation during the drying stage, and improve the long-term product stability (Robinson, 2016). To avoid any potential confusion to the readers, the term '**lyoprotectant**' is now changed to '**protectant**' throughout the manuscript.

- ***Comment 3:***

For liposome nanocrystal, perhaps the most important feature is the dissolution profile, which should be measured. Also, TEM images should be provided to show the liposome remains intact to support the drug release data, at least on the optimized formulation before and after spray drying.

We agree with the reviewer to include the *in vitro* drug release data of the optimized formulations. Under Section **2. Materials and methods,** the following section was added**:**

2.9.3. In vitro assay of Cf release from liposomes

Under Section **3. Results and Discussion**, the following section was added:

3.6.3. In vitro assay of Cf release from liposomes

Please refer to the revised manuscript for more details.

Cryo-TEM images were reported in the supplementary file for the 45 spray dried samples.

- ***Comment 4:***

In the HPLC method, the thermal decomposition products of ciprofloxacin should be evaluated,

at least on the final product. Currently, it is not clear if thermal decomposition plays a role in product quality. There are many established analytical methods for this. (i.e. Aksoy, B., Küçükgüzel, İ. & Rollas, S. Development and Validation of a Stability-Indicating HPLC Method for Determination of Ciprofloxacin Hydrochloride and its Related Compounds in Film-Coated Tablets. Chroma 66, 57-63 (2007).https://protect-au.mimecast.com/s/a_a5C4QOPEiWqAzEUVFRoa?domain=doi.org).

In the current formulation, Cf is encapsulated inside liposomal vesicles with sugar protectants. In the literature, ciprofloxacin was reported to have a very high thermal stability up to 200 °C (Svahn and Björklund, 2015). Throughout the present study, the drying temperature was ranging between 50 and 80 °C. Since the Cf in our study was not exposed directly to strong heat during the process, degradation is very unlikely to have occurred. To confirm, we have conducted further tests by exposing the powder Cf to dry heat at 60 ° C following the thermal degradation method reported in the paper that was suggested by the reviewer. HPLC analysis showed that the % area of Cf did not change significantly (*p*-value = >0.9999) and so were the impurities (*p*-value = >0.9999) as compared to the fresh powder sample (**Table 1**). Furthermore, when we exposed the aqueous solution of Cf to 90 °C for about 30 min, the HPLC analysis showed a non-significant change in the % area of each peak (*p*-value = >0.9999) in comparison to the fresh powder sample.

Table 1: Percent area (% Area)* of impurities and Cf in various samples analyzed by HPLC.

Peaks (impurities and Cf)	Retention time (minutes)	% Area*		
		Fresh sample	Thermally decomposed	
		Powder	Powder	Aqueous solution
Peak 1	3.98	0.05	0.05	0.01
Peak 2	6.87	0.14	0.13	0.19
Cf Peak	12.42	99.81	99.83	99.80

***** Percent area (% Area) was calculated by dividing each peak area by the area of the total peaks and multiplied by 100 to convert to a percentage.

Reviewer #2:

Manuscript should revise by considering following points.

- ***Comment 1 & Comment 3:*** Language should be updated in scientific form throughout the manuscript. Results and discussions should modify more scientific way with proper references.

The whole manuscript was thoroughly revised and modified to address the above comments. Please refer to the revised version of the manuscript for more details. The following references were added to strengthen the relevant discussion parts of the manuscript:

1- Weers, J., 2015. Inhaled antimicrobial therapy–barriers to effective treatment. Advanced Drug Delivery Reviews 85, 24-43.

2- Cipolla, D., Blanchard, J., Gonda, I., 2016a. Development of liposomal ciprofloxacin to treat lung infections. Pharmaceutics 8, 6.

3- Chan, H.-K., Clark, A.R., Feeley, J.C., Kuo, M.-C., Lehrman, S.R., Pikal-Cleland, K., Miller, D.P., Vehring, R., Lechuga-Ballesteros, D., 2004. Physical stability of salmon calcitonin spray-dried powders for inhalation. Journal of pharmaceutical sciences 93, 792-804.

4- White, S., Bennett, D.B., Cheu, S., Conley, P.W., Guzek, D.B., Gray, S., Howard, J., Malcolmson, R., Parker, J.M., Roberts, P., 2005. EXUBERA®: pharmaceutical development of a novel product for pulmonary delivery of insulin. Diabetes technology & therapeutics 7, 896-906.

5- Barenholz, Y.C., 2012. Doxil®—the first FDA-approved nano-drug: lessons learned. Journal of controlled release 160, 117-134.

- ***Comment 2:*** Experimental mathematical equations should be added in results with explanation.

The experimental mathematical equations of each response for each protectant were listed in **Table 8** in the manuscript. The relationship between the responses and the factors was thoroughly explained in the following sections of the manuscript:

3.3. Estimation of quantitative effects of the factors

3.4. Three-dimensional (3D) response surface plots

- ***Comment 4:*** In conclusions, add how BBD is most suitable in experimental design.

  The BBD was chosen according to the basis explained in the fourth paragraph of the Introduction Section of the manuscript. However, we agree with the reviewer to emphasize again the usefulness of such a design in achieving the purpose of this study. Thus, the below sentences are added to the Conclusion Section of the manuscript:

  In conclusion, the Box–Behnken design of experiments is an economical way to extract the maximum amount of complex information for the CNL production method, with the process characterization and optimization achievable with a relatively small number of experimental runs in a short time.

- ***Comment 5:*** All references should updated with proper uniform format.

  All the references were thoroughly revised in the References Section of the manuscript to confirm their format uniformity.

References:

Emami, F., Vatanara, A., Park, E.J., Na, D.H., 2018. Drying technologies for the stability and bioavailability of biopharmaceuticals. Pharmaceutics 10, 131.

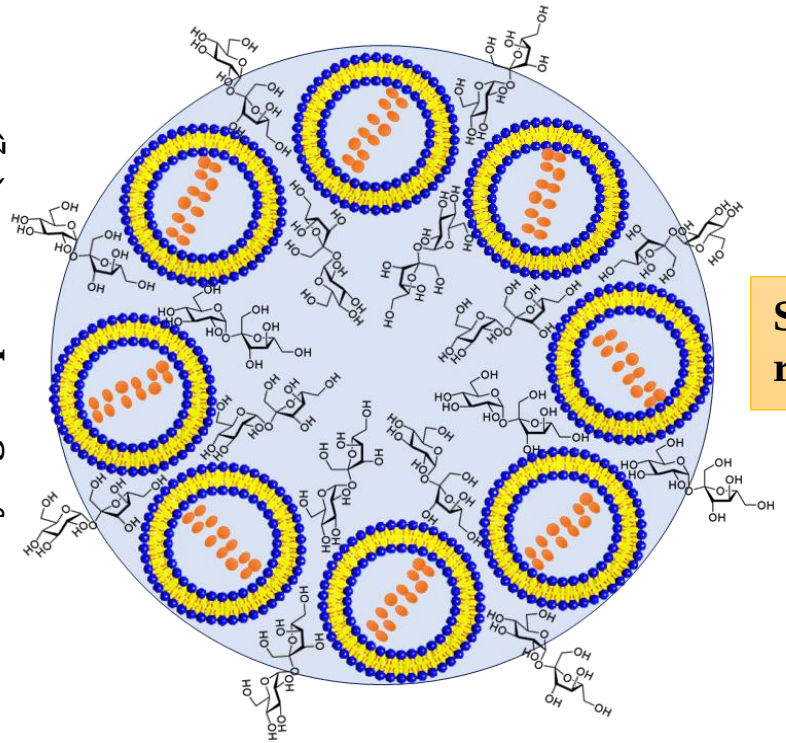
Robinson, T.D., 2016. Compositions and methods for atmospheric spray freeze drying. Google Patents.

Svahn, O., Björklund, E., 2015. Thermal stability assessment of antibiotics in moderate temperature and subcriticalwater using a pressurized dynamic flow-through system. International Journal of Innovation and Applied Studies 11, 872-880.

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_1^2 + b_5x_2^2 + b_6x_3^2 + b_7x_1x_2 + b_8x_1x_3 + b_9x_2x_3 + E$$

Protectant amount (x_1)

Drying temperature (x_2)

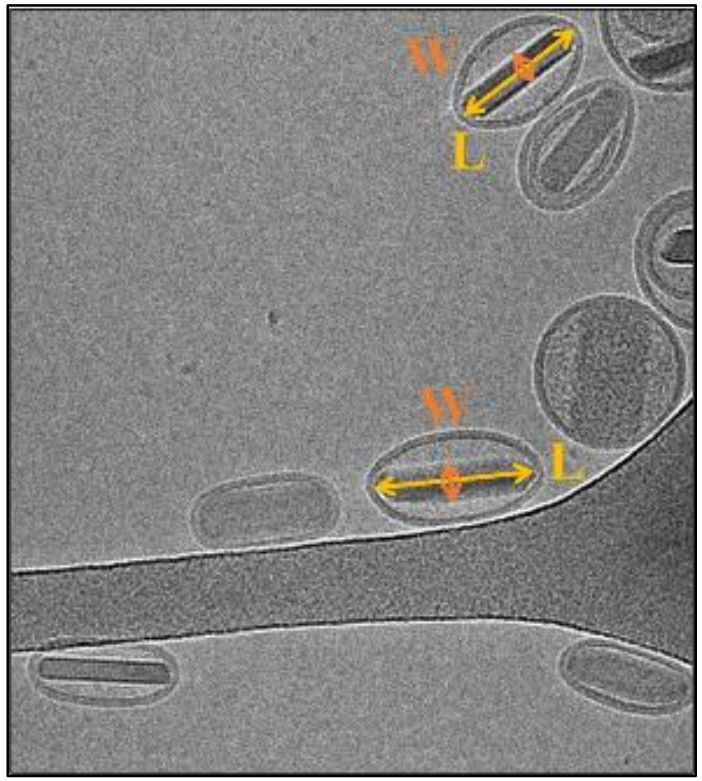


Spray gas flow (x_3)

Spray dried powder reconstituted in saline

Liposomes size (y_1)

Encapsulation efficiency (y_2)



Nanocrystals dimensions (y_4)

Surface zeta potential (y_3)

Sprayed droplet **Liposomal vesicle** **Sucrose molecule** **Ciprofloxacin molecule** **Ciprofloxacin nanocrystal**

Research paper

Modeling of a spray drying method to produce ciprofloxacin nanocrystals inside the liposomes utilizing a response surface methodology: Box-Behnken experimental design

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Abstract

Spray drying was previously used to modify the physical form of the encapsulated ciprofloxacin drug to produce ciprofloxacin nanocrystals inside the liposomes (CNL). The purpose of the present study was to optimize CNL powder production by evaluating the response surface via design of experiments (DoE). Using the Box–Behnken (BB) design, the study independent variables were the protectant type (sucrose, trehalose or lactose), protectant amount, drying temperature, and spray gas flow. Individual spray drying experiments were performed at various set points for each variable followed by characterization of the produced powders. Liposomal particle size, drug encapsulation efficiency (EE%), liposomal surface zeta potential, and nanocrystal dimensions were the design dependant variables. By applying the least square regression method on the experimental data, mathematical models were developed using the mathematical software package MATLAB R2018b. Model reliability and the significance of the model's factors were estimated using analysis of variance (ANOVA). The generated CNL powders showed spherical to elliptical liposomal vesicles with particle sizes ranging from 98 to 159 nm. The EE (%) ranged from 30 to 95% w/w while the zeta potential varied between -3.5 and -10.5 mV. The encapsulated ciprofloxacin nanocrystals were elongated cylindrical structures with an aspect ratio of 4.0 - 7.8. Coefficients of determination ($R^2 > 0.9$) revealed a good agreement between the predicted and experimental values for all responses except for the nanocrystal dimensions. Sucrose and lactose were superior to trehalose in protecting the liposomes during spray drying. The amount of sugar significantly affected the characteristics of the CNL powders (p-value < 0.05). In conclusion, the DoE approach using BB design has efficiently modelled the generation of CNL by spray drying. The optimum processing conditions which produced high drug encapsulation (90%) after formation of nanocrystals and a vesicle size of ~125 nm utilized 57% (w/w) sucrose, an 80°C inlet temperature, and an atomization rate of 742 L/hr.

Keywords:

Spray drying; ciprofloxacin nanocrystals; liposomes; response surface methodology; Box-Behnken design; modeling

Abbreviations

ANOVA, analysis of variance; BB, Box–Behnken; Cf, ciprofloxacin; CNL, ciprofloxacin nanocrystals inside liposomes; Cryo-TEM, cryogenic transmission electron microscopy; DC, drug content; DLS, dynamic light scattering; DoE, design of experiments; DPI, dry powder inhaler; EE, encapsulation efficiency; FPF, fine particle fraction; HBS, HEPES buffered saline; HPMC, hydroxypropyl methylcellulose; HSPC, hydrogenated soy phosphatidylcholine; MMAD, mass median aerodynamic diameter; NGI, next generation impactor; NTM, non-tuberculous mycobacteria; SD, standard deviation; T_g, glass transition temperature; T_m, gel-liquid transition temperature; VIF, variance inflation factor.

1. Introduction

Drug nanocrystals inside liposomes are attracting the attention of the pharmaceutical industry due to their unique properties. With the addition of a dissolution step, vesicular systems encapsulating drug nanocrystals will possess a slower drug release rate, maintain a higher drug loading and/or allow improved targeting to specific cells or tissues (Cipolla et al., 2016c). The engineered drug nanocrystals can be administered by various routes, including aerosol delivery. Inhaled antibiotic formulations utilizing liposome-encapsulated drug nanocrystals will possess a longer residence time due to their slow dissolution rate (Li et al., 2018a). Less frequent dosing may lead to improved patient convenience. Additionally, these systems may be better able to target intracellular infections like non-tuberculous mycobacterium (NTM) residing in pulmonary macrophages because of the slower antibiotic release rate allowing time for the macrophages to phagocytose the liposomes (Blanchard et al., 2014; Blanchard et al., 2018; Gonda et al., 2019).

The ability to convert the encapsulated drug state from a soluble to a solid form without changing the liposome composition would provide opportunities to attenuate the release profile and develop personalized approaches to treatment (Cipolla et al., 2014b). For some drugs, nanocrystals form spontaneously after being entrapped into liposomes; e.g., doxorubicin (Lasic et al., 1992; Li et al., 1998), topotecan (Abraham et al., 2004), and vinorelbine (Zhigaltsev et al., 2006). However, other drugs remain as a supersaturated solution within liposomes. In several liposomal formulations designed for treating lung infections via inhalation,

ciprofloxacin (Cf) is not in crystalline form (Drummond et al., 2008; Maurer-Spurej et al., 1999; Webb et al., 1998). One mechanism to induce formation of Cf nanocrystals was utilizing a simple freeze-thaw process of the supersaturated liposomal formulations. This process created ice crystals inside the vesicles which served as nucleation sites for drug crystallization (Cipolla et al., 2016c; Li et al., 2018b). Spray drying, an established technique for transforming solutions or liquid dispersions into dry powders, was recently exploited to produce Cf nanocrystals within liposomal vesicles (Khatib et al., 2020). The success of this process is dependent on many factors. Liposomes encounter multiple stresses (e.g. mechanical and osmotic stresses) during spray drying, thus the use of stabilizing agents (protectants) is of paramount importance for protection purpose. Although the widely used protectants for spray drying liposomes are disaccharides; e.g. sucrose, trehalose and lactose (Ingvarsson et al., 2011), only one sugar, sucrose, was investigated by Khatib et al. (2020), and the protective efficiency of other sugars has yet to be explored. Both the type and the amount of a protectant may determine the level of liposomal protection during spray drying (Ingvarsson et al., 2011; Khatib et al., 2019; Khatib et al., 2020). In addition, the spray drying parameters, particularly the inlet air temperature and spraying gas flow for atomization, influence the magnitude of the stresses imposed on the liposomal vesicles during processing (Lo et al., 2004; Wessman et al., 2010). In the initial investigation (Khatib et al., 2020), those two parameters were kept constant so there is a limited understanding of their influence. Optimization of the spray drying process for the generation of CNL has not been studied or reported.

The objective of the present study was to characterize the parameters which have a significant effect on the characteristics of CNL powders obtained by spray drying. In this study, the relationship between four response variables (liposomes particle size, zeta potential, encapsulation efficiency, and Cf nanocrystal dimensions) and three quantitative parameters (amount of protectant, inlet temperature, and spray gas flow for atomization) were determined using design of experiments (DoE) approach.

Box–Behnken (BB) is an experimental design approach that requires relatively few design points and experiments to be conducted to produce a comprehensive dataset for analysis (Kincl et al., 2005; Ragonese et al., 2002). Moreover, this design is more favorable experimentally and economically as compared to other designs due to its use of three instead of five levels for each factor (Montgomery, 2017; Ragonese et al., 2002). The aim was to use the BB design to obtain polynomial mathematical equations and response surface plots to

determine the combination of spray drying physiochemical parameters to generate CNL powders with predictable characteristics.

In the following sections, the three-level three-factorial BB experimental design for investigation, characterization, and optimization of spray drying parameters for the production of CNL inhalation powders is described.

2. Materials and methods

2.1. Materials

Liposomes encapsulating 50 mg mL^{-1} ciprofloxacin hydrochloride dispersed in an aqueous pH 6.0 histidine buffer were produced by Exelead (Indianapolis, IN, USA) and Northern Lipids Incorporated (Burnaby, BC, Canada) and provided by Aradigm Corporation (Newark, CA, USA). Isoleucine, magnesium stearate, sucrose, trehalose, sodium chloride, adult bovine serum and triethylamine (TEA) were all of analytical grade and bought from Sigma-Aldrich (Castle Hill, New South Wales, Australia). Lactose was obtained from DFE Pharma (Goch, Germany) and deionized water from Modulab Type II Deionization System (Continental Water System, Sydney, Australia). HPLC grade methanol was purchased from Thermo Fischer Scientific (Victoria, Australia) and HEPES, free acid from Dojindo, China. Nanosep Omega centrifugal filtration devices, 10k molecular weight were supplied from Pall Australia Pty Ltd, (Victoria, Australia). An Osmohaler® inhaler was obtained from Pharmaxis Ltd. (Frenches Forest, Australia) and size 3 hydroxypropyl methylcellulose (HPMC) capsules from Capsugel (West Ryde, Australia).

2.2. Experimental design

In general, the BB design approach is used to obtain a quadratic response surface and construct a second-order polynomial model. The design is composed of replicated centre points (three experiments) and a set of points lying at the midpoints of each edge of the multidimensional cube that define the region of interest (twelve experiments). Also, the design can be structured as three blocks of four experiments each representing a full two-factor factorial design with the third-factor level set at zero (**Fig. 1**)(Souza et al., 2005). Overall, a total of fifteen experiments is required. The model output is of the following form (**Eq.1**):

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_1^2 + b_5x_2^2 + b_6x_3^2 + b_7x_1x_2 + b_8x_1x_3 + b_9x_2x_3 + E$$

where y is the response, b_0–b_9 are the regression coefficients, x_1, x_2 and x_3 are the factors studied and E is the error term. An orthogonal design like the BB approach leads to evenly spaced factor levels and is encoded with low, medium and high settings; i.e. −1, 0 and +1 (Kincl et al., 2005).

In this study, a three-level, three-factorial BB experimental design was implemented to examine the effects of certain independent variables on the dependant responses, so as to characterise and optimize the spray drying process for the generation of CNL dry powders. Three protectants (sucrose, trehalose, and lactase) were considered to stabilize the liposomes during spray drying and they were each tested separately. These sugars were chosen due to their historical use to stabilize liposomes during drying (Ingvarsson et al., 2011). For each sugar type, a total of 15 experimental runs were conducted (**Table 1**). The initial spray drying studies on liposomal ciprofloxacin identified the factors and the settings of factor levels (Khatib et al., 2019; Khatib et al., 2020). The factors studied in the BB experimental design were: protectant amount (x_1), inlet drying temperature (x_2) and atomization or spray gas flow (x_3). The factor levels for protectant amount were chosen in accordance with the experimental values that produced acceptable drug encapsulation efficiency (EE) and drug content (DC) from previous studies. Thus, in this study 57% w/w was chosen as the highest value of factor x_1 in order not to go below 12% w/w DC, whereas 25 % w/w was the lowest value of this factor so the drug EE will remain above 30% w/w (Khatib et al., 2019; Khatib et al., 2020).

According to our experience in spray drying, the lowest inlet temperature that can be set stably to achieve efficient drying is 50°C. On the other hand, temperatures which are 20°C higher than the sugar glass transition temperature (T_g) may produce particles with a sticky surface forming paste-like structures after spray drying, which is not suitable for powders intended for pulmonary delivery (Muzaffar et al., 2015; Rahman, 1999). In the literature, the T_g of sucrose, trehalose and lactose are 62, 100 and 101°C, respectively (Roos, 1993). Therefore, the lowest and the highest values of the factor x_2 were 50 and 80°C, respectively.

The factor levels for atomization (x_3) were chosen according to the atomization settings of the Büchi spray dryer and the corresponding droplet size (**Table 2**). The minimum and maximum atomization values produced a spray droplet size difference of 16.8 μm which may be sufficient to explore the atomization effect on the characteristics of the CNL inhalation powders that were generated. With the minimum and maximum values identified, the middle

values are simply an average of the extreme values. Thus, the middle values for factors x_1, x_2 and x_3, are 41% w/w, 65°C and 648 L/hr, respectively.

The selected responses are liposome size (y_1), drug EE (y_2), liposome zeta potential (y_3) and ciprofloxacin nanocrystal dimension (y_4). **Table 3** shows the chosen factors and the settings of the factor levels. The responses studied and the constraints selected are presented in **Table 4.** The statistical software used to evaluate the experimental design and construct the mathematical models are MATLAB R2018b (MathWorks, MA, USA) and Design-Expert v12 (Stat-Ease, MN, USA). The coefficients; i.e., the main effect (bi) and two factor interactions (bij) were estimated from the experimental results by applying the least square regression method.

2.3. Spray drying method

An aqueous dispersion of liposomal ciprofloxacin at 50 mg mL^{-1} was mixed with 25, 50 or 100 mg mL^{-1} protectant solution at a 1:2 v/v ratio and 1:1 v/v with deionized water. The solid concentration of the final dispersions ranged between 8.6 and 10.6 mg mL^{-1} and the dispersion was fed into a spray dryer (B-290 mini spray-dryer, Büchi Flawil, Switzerland) while under continuous stirring. Feed rate and aspirator settings were kept constant for all runs at 1.4 mL/min and 35 m^3/h, respectively. Inlet air temperature and atomizer settings were changed in each run according to the coded values stated in **Table 1** and their actual corresponding values in **Table 3.** After spray drying, the powder was collected and stored in a dry container (<15% RH) protected from light at ambient temperature (~ 23°C).

2.4. Particle size distribution and surface zeta potential of liposomes: Dynamic light scattering (DLS)

Dry powder formulations were reconstituted in saline followed by dilution to 10 mM NaCl for particle size and zeta potential measurements. The samples were measured using a Malvern Zetasizer Nano ZS (Malvern, UK) with disposable folded capillary cell (DTS1070), with the following instrument parameters: temperature of 25°C, viscosity of 0.887 cP, refractive index of 1.34, backscatter angle of 173°, and a run time of 5 min. Smoluchowski model was used for zeta potential measurements and Henry`s function (F(ka)) value was set at 1.5. Three samples of each spray-dried powder were tested, with the data reported as mean ± SD.

2.5. Drug encapsulation efficiency (EE)

The percentage of drug encapsulated within the liposomal vesicles was determined after reconstituting the spray-dried powder in saline. Samples of Cf at a total concentration of ~1 mg mL^{-1} were filtered using Nanosep Omega centrifugation devices with modified polyethersulfone membrane filters of 10,000 molecular weight cut-offs (Cipolla et al., 2014b). The filtration was performed by transferring 400 µL of each sample followed by centrifugation for 18 min at 10,000 rpm (6,700 × *g*). The filtrate was diluted 20 times with deionized water and loaded in the HPLC (described below) to quantify the unencapsulated Cf (free Cf). Another 1 mL aliquot of each sample was diluted with 9 mL of 80% v/v methanol to solubilize the liposomes, then centrifuged for 15 min at 13,400 rpm (12,100 × *g*). The filtrate was diluted 4-fold with deionized water, then analysed using HPLC to measure the total amount of Cf in the samples. The percentage of the encapsulated drug was calculated for three measurements using the equation below:

EE% = [(Total drug amount – Free drug amount) / Total drug amount] ×100% (**Eq. 2)**

2.6. Nanocrystals dimensions: Cryogenic transmission electron microscopy (cryo-TEM)

Dry powders were reconstituted in saline to a total Cf concentration of ~1 mg mL^{-1}. Samples were loaded at 4 µL aliquots into a glow discharged Lacey formvar/carbon grid (TED PELLA, USA) mounted inside a chamber controlled at 4°C and 85% RH. The samples were blotted once with filter paper for 3 s and vitrified by plunging into liquid ethane using a Leica EM GP device (Lecia Microsystem, Germany). The vitrified samples were stored in a liquid nitrogen dewar prior to analysis by cryo-TEM. A Talos Arctica transmission electron microscope (Thermo Fisher Scientific, USA) operating at 200 kV was utilized for imaging the stored samples (Cipolla et al., 2016c; Khatib et al., 2019). The collected images were used to confirm the formation of Cf nanocrystals. Also, the length (L) and width (W) of the Cf nanocrystals inside the liposomes were analysed manually using ImageJ software (Schindelin et al., 2012). Data presented were representative of at least 100 individual measurements on more than 6 different micrographs (mean ± SD). Both the length and width for each nanocrystal were measured between two points lying on the nanocrystal edges across from each other (**Fig. 2**). From these numbers, the length/width (L/W) ratio of each nanocrystal can be calculated where a value far from 1 represents an elongated cylindrical nanocrystal while a value closer to 1 represents a less elongated cubic nanocrystal (Cipolla et al., 2016c).

2.7. Quantification method of Cf: High-performance liquid chromatography (HPLC)

The amount of Cf in the samples was determined by applying the HPLC method stated previously (Ong et al., 2014). The stationary phase of the system was Phenosphere-Next C-18 column (5 mm, 4.6 × 150 mm, Phenomenex, USA) at 35°C temperature. An isocratic method utilized a mobile phase of 0.5 % TEA, pH 3.0 buffer and 100 % methanol (78: 22 v/v) at a flow rate of 0.9 mL min^{-1}. The measuring wavelength of Cf was set at 277 nm on the system UV detector. The concentration of Cf was expressed in terms of Cf HCl.

2.8. Optimization of formulations and Validation

In the study, responses were optimized using a numerical optimization called desirability function approach (Derringer and Suich, 1980). In this approach, a specific goal was assigned to each response. A partial desirability function is linked with an individual response, where value 0 is allocated to an undesired response while value lies between 0 and 1 is assigned to an acceptable response. The value between 0 and 1 indicates the closeness of the response to its target value. Therefore, desirability function helps in ascertaining the appropriate independent factor values in the design space that accomplishes the set goals for the dependant variables. In our study, Design-Expert v12 was utilized to obtain the maximum desirability value after assigning desired goals to the responses. To validate the ability of the polynomial equations to predict responses, spray drying trials of liposomal ciprofloxacin dispersions using the optimum parameters were carried out for each protectant followed by characterization of the resulted powders.

2.9. Evaluation of optimized formulations for respiratory delivery and controlled drug release

To evaluate the suitability of the optimized formulations for respiratory delivery, formulations were prepared using the optimized conditions with the addition of specific excipients to serve as moisture protectants and dispersibility enhancers. According to our experience, the addition of 2% w/w magnesium stearate and 5% w/w isoleucine improved the characteristics of the spray-dried CNL powders (Khatib et al., 2019; Khatib et al., 2020). Thus, these excipients were added to the aqueous dispersions prior to spray drying. The obtained powders were collected and characterized using the following methods.

2.9.1. Moisture content: Thermogravimetric analysis (TGA)

The residual moisture content of the optimized formulations was determined by thermogravimetric analysis (TG/SDTA 851e, Mettler-Toledo, Germany). Alumina pans were loaded with 5-10 mg of each formulation and heated from 30 to 150 °C at a rate of 10 °C/min under nitrogen gas. Water content was represented as the percent drop in mass observed between 30 and 100 °C. The data were reported as mean and SD of three measurements for each formulation.

2.9.2. In vitro aerosol performance

A next generation impactor (NGI, Copley, Nottingham, UK) attached to a mouthpiece adapter and a USP throat (dry USP induction port) was utilized to evaluate the aerosol performance of the optimized formulations. Each formulation (30±1 mg) was weighed into a size 3 HPMC capsule which was then loaded to an Osmohaler® inhaler device for powder dispersion under ambient conditions. A pressure drop of about 4 kPa was generated across the device when four liters of air were drawn through the inhaler at 100 L min^{-1} for 2.4 s (Tiddens et al., 2006). After dispersion, the total recovered mass was measured by washing the capsule, inhaler, adapter, throat, and stages 1–8 of the NGI with 80% methanol to collect deposited powders for HPLC analysis. The percent mass of powder exiting the capsule and device relative to the total recovered mass was considered the emitted dose (ED). The fine particle fraction (FPF) was calculated as the percent of the total recovered powder mass with a particle size ≤ 5 µm. Mass median aerodynamic diameter (MMAD) was the particle size below which 50 % wt. of the particle population lies.

2.9.3. In vitro assay of Cf release from liposomes

According to a previous validated method for Cf release from liposomes (Cipolla et al., 2014a), the optimized formulations were reconstituted and a control (original aqueous Cf liposomal dispersion) was diluted in saline. For further dilutions, HEPES buffered saline (HBS: 20 mM HEPES, 145 mM NaCl, pH 7.4) was used to obtain a final concentration of around 50 µg mL^{-1} of Cf. At the beginning of the release study, a chilled (2–8 °C) adult bovine serum (Sigma-Aldrich, Castle Hill, New South Wales, Australia) was added in equal volume to the prepared dispersions followed by withdrawing samples at time zero. Dispersions were loaded into a shaking water bath (Labec J-SWB60, Marrickville, Australia) at 37 °C and 150 rpm. Samples were withdrawn at Tn = 30, 60, 120, 240, 480 and 600 min of incubation and

immersed immediately in an ice-water bath followed by adding an equal volume of chilled (2–8°C) HBS to eliminate further release of Cf. Nanosep centrifugal devices were loaded with 400 µL of each sample and centrifuged at 10,000 rpm for 18 min to separate released Cf from the encapsulated Cf. Filtrates were analysed via HPLC to quantify the amount of released Cf. The expected loss of drug in the filter in the presence of the serum was compensated by normalizing the calculated values with a correction factor of 0.93 (Cipolla et al., 2014a). The total Cf amount was determined by following the method described in **Section 2.5**. The percent Cf released was the amount of the released Cf relative to the total Cf amount in the sample. A normalized percentage of release was calculated due to the presence of free drug at Tn= 0min; hence, the release of Cf at Tn (Tn-Tn= 0min) was divided by the total possible release (100-Tn= 0min) and then converted to a percentage: 100* (Tn-Tn= 0min)/(100-Tn= 0min) (Cipolla et al., 2016b).

3. Results and Discussion

3.1. The results of BB Experiments

The spray dried powders were assessed for liposome size (y_1), drug encapsulation (y_2), zeta potential (y_3), and the dimensions of the encapsulated ciprofloxacin nanocrystals (y_4) as summarized in **Table 4**. The experimental output values ($y_1, y_2, y_3,$ and y_4) for each of the fifteen runs are listed in **Tables 5 -7** for sucrose, trehalose and lactose, respectively. Avoiding liposome disruption and premature loss of drug during spray drying is a key metric. A low encapsulation efficiency would mean that a higher proportion of the drug would be immediately available in the lung, and no longer contributing to a sustained release profile necessary to maintain drug concentrations above the minimum inhibitory concentration to combat the pathogen (Weers, 2015)(Weers et al., 2015). Because of the wide variation in encapsulation efficiency (y_2), the runs with the experimental parameters that generated powders with the highest drug encapsulation efficiency are highlighted in bold in these tables. These conditions all utilized the highest level of sugar protectant (x_1), 57% (w/w). These runs also produced spray dried powders with acceptable values for the other parameters of liposome size and zeta potential.

The drug encapsulation efficiency varied widely across the BB design space with the lowest values of ~30% observed for each of the three protectants while the highest value of ~95% was only achieved for sucrose or lactose. The observation that sucrose and lactose were superior to trehalose is consistent with that reported previously for stabilization of liposomal

ciprofloxacin to freeze-thaw (Cipolla et al., 2016). In those studies, trehalose only partially preserved liposome integrity versus sucrose which maintained complete retention of liposome integrity. The inferior performance of trehalose in comparison to sucrose may be specific to the type of lipid that comprises the liposomal membrane which in this case is HSPC and cholesterol; sucrose may have a greater capability than trehalose to form hydrogen bonds with HSPC, reducing the number of water molecules associated with each liposome, and thus stabilizing the liposomes during spray drying (Cipolla et al., 2016c; Ingvarsson et al., 2011).

The liposomes in the spray dried powders after reconstitution were spherical to elliptical vesicles with a particle size ranging from 98 – 159 nm for all protectants. The liposome size prior to spray drying was between 70-100 nm which had already been established to provide an acceptable shelf life in solution (Cipolla et al., 2016a; Cipolla et al., 2016c; Khanal et al., 2020). A deviation from that size range may increase the likelihood for unacceptable stability after reconstitution. Thus, for these studies, a target liposome size near 100 nm was chosen. The eight conditions which produced powders with the highest amount of drug retention had median vesicle sizes ranging from 109 to 124 nm.

Negative values for liposomal surface zeta potential were observed across all the experimental runs for the three protectants. The zeta potential values for the spray dried powders after reconstitution varied between -3.5 and -10.5 mV. A value of around -10 mV was obtained for the original liposomal dispersion which had an acceptable shelf-life and so was considered optimum (Cipolla et al., 2016a), and similar values were reported for other liposomal dispersions composed of HSPC phospholipids (Chen et al., 2012). Thus, a zeta potential close to -10 mV was taken to be indicative of a stable liposomal preparation.

Formation of nanocrystals within the liposomes was confirmed using the cryo-TEM images (**Figs. (1–3) S**) and their dimensions were measured using Image J software. Elongated nanocrystals were generated in all the experimental runs for the three protectants. For the three protectants, the lowest calculated length to width ratio was 4.0 while the highest was 7.8, implying that rod-like or cylindrical nanocrystals were formed regardless of the applied spray drying conditions.

3.2. Construction of the second-order model and analysis of variance (ANOVA)

From the experimental results listed in **Tables 5-7** and **Eq. 1**, the second-order response functions representing liposome particle size, drug EE, liposome zeta potential and nanocrystal dimensions can be expressed as a function of protectant amount, inlet drying temperature and

atomization. The relationships between the four responses (y_1, y_2, y_3 and y_4) and the factors obtained using computer simulation programming (MATLAB R2018a) are listed in **Table 8**.

The model reliability was verified by ANOVA and multiple correlation tests (R^2) were conducted using the statistical software (Design-Expert version 12). The quadrant model was concluded to be significant for all responses except the nanocrystal dimensions (y_4) as shown in **Table 9**. For all responses except for y_4, the p-value was < 0.05. Thus, the three independent variables, x_1, x_2 and x_3, had a significant effect in predicting the responses for y_1, y_2 and y_3. In addition, the multicollinearity between the independent variables was examined by the variance inflation factor (VIF). The VIF value was 1 for all of the variables in the quadratic model indicating that there is no correlation between them. In general, VIF values of less than 10 are considered tolerable. The experimental and predicted values of responses are given in **Table 1S** and the coefficients of determination (R^2) for all responses were calculated and presented in **Table 9**. For all protectants, the R^2 values were close to 1 for the responses y_1, y_2 and y_3 (but not y_4 which was not significant) and in reasonable agreement with the adjusted R^2 for responses y_1, y_2 and y_3 which indicates the goodness of fit to the response variables.

3.3. Estimation of quantitative effects of the factors

The coded coefficients (e.g., b_1, b_2, etc) and their p-values are shown in **Table 10**. A high coefficient value associated with a p-value of less than 0.05 indicates the intrinsic effect of the factor on the response. A factor (e.g., b_1) with a positive value (e.g., 5.59 for y_1 for sucrose) implies that there will be an increase in the response (y_1, or liposome particle size) with an increase in the factor value (x_1, or amount of sucrose) while a negative factor value will imply a decrease in the response with an increase in the factor value.

From **Table 10**, response y_1 (liposome particle size) for the three protectants was significantly affected to a negative extent by the quadratic term of the protectant amount (x_1^2), with sucrose being also affected to a positive extent by the protectant amount (x_1) and trehalose to a positive extent by the atomization (x_3). The response y_2 (EE) of the three protectants was significantly affected to a positive extent by the protectant amount (x_1) and to a negative extent by its quadratic term (x_1^2). The EE of lactose was also significantly affected to a positive extent by the atomization (x_3). Response y_3 (zeta potential) of the three sugars is significantly affected to a negative degree by the protectant amount (x_1) while trehalose was also affected to a negative degree by its quadratic term (x_1^2). The values indicate no significant effects of any

factor on the response y_4 (nanocrystal dimension) except for trehalose which was affected positively by the protectant amount (x_1).

The major factor affecting the stability of the liposomes during the process was the protectant amount. Hauser and Strauss (1987) reported that spray drying liposomes with zero sucrose led to aggregation and fusion of liposomal vesicles in addition to a 65 – 80% drop in entrapment efficiency. Consequently, protectants; i.e. sugars, became an integral part of the subsequent studies of liposomes encapsulation efficiency and membrane integrity during spray drying. Interaction effects (cross-product terms) were not found to be significant for all four responses. Inlet temperature (x_2) was not a significant factor with respect to any of the responses for all the tested protectants. These liposomes encapsulate a hydrophilic drug in their aqueous core, and the drug may diffuse out with water during drying. In the present study, the outlet temperature was varied from 32 – 52°C. The T_m of HSPC, which is the main component of the liposomes under evaluation, is around 55°C (Alavizadeh et al., 2014). Goldbach et al. (1993a; 1993b) observed that more leaky liposomes result when dried at a temperature higher than their gel-liquid transition temperature (T_m). Hence, the influence of drying temperature on the stability of the spray-dried liposomes was not a major factor since all drying temperatures were below 55°C.

3.4. Three-dimensional (3D) response surface plots

The measured responses were plotted as three-dimensional (3D) graphs in accordance with the model polynomial functions to evaluate the change of the response surface. These plots are also convenient for further exploration of the relationship between the dependent and independent variables. Since the design is a three factors model, the values of two factors are varied while the third is held constant for each diagram. Hence, a total of 12 response surface diagrams were produced for each protectant. **Figs. 3–5** show the 3D response surface plots associated with the significant factors for three responses (y_1, y_2 and y_3) of the sucrose protectant. Plots of the other protectants are presented in **Figs. (4–9) S** and plots associated with non-significant factors are presented in **Figs. (10–14) S**. The dominant factor was the protectant amount which was directly proportional to the encapsulation efficiency and inversely proportional to the zeta potential (**Fig. 3** and **4**).

Liposomal particle size increased as the sugar amount decreased, until a certain threshold (approximately 41% by weight) after which the vesicle size dropped and returned to its original size (**Fig. 5**). Multiple factors can affect the liposomal structure during spray drying;

i.e., osmotic stress, lipid properties and particle to particle interactions. Structural re-arrangement may occur because of one factor or a combination of multiple factors (Wessman et al., 2010). It is not straightforward to understand why liposomes may display inconsistent behaviour upon drying with different protectant amounts. **Fig. 7** represents a possible scenario of events occurring during the drying of a sprayed liposomal ciprofloxacin dispersion. Sprayed water droplet of the liposomal dispersion containing a high amount of sugar (e.g., sucrose) will be crowded by both sucrose molecules and liposomal vesicles (**Fig. 7A**). Those vesicles are mainly located on the droplet surface and they are fully loaded with drug molecules. Sucrose molecules may reduce vesicular interactions by forming hydrogen bonds with the phosphate groups of the HSPC lipid and occupying the spaces between the adjacent vesicles. Although drying will increase the sucrose concentration on the outside of the vesicles, the concentration of encapsulated ciprofloxacin inside is elevated too. Thus, the osmotic pressure differential across the liposomal membrane may not change substantially and as a result, liposomal vesicles tend to retain their original size upon rehydration. Decreasing the amount of the sucrose in the spray dried dispersion will reduce protection and stabilization of the liposomal vesicles, increase their potential to interact and allow some drug leakage (**Fig. 7B**). All these events will contribute to producing larger liposomal structures when the spray-dried powder is reconstituted. A further decrease (< 41% w/w) in the amount of incorporated sucrose renders the sprayed droplets less crowded (**Fig. 7C**). The opportunity for particles to come into contact and interact will decrease, while drug leakage due to less sugar stabilization may increase substantially. Because of the presence of sucrose and drug on the outside, the osmotic pressure forces the encapsulated water to diffuse out, and the size of the liposomes is thereby reduced upon rehydration (Wessman et al., 2010). In the case of trehalose, a high sugar amount was also associated with the highest elongation in the cylindrical nanocrystals (**Fig. 6)** which was correlated with the highest encapsulation efficiency value.

3.5. Optimization of formulations and validation

The desirability function was probed using Design-Expert 12 software to acquire the optimized formulation. The constraints were set for all the responses as shown in **Table 4**. The factors were set in the range as depicted in **Table 3** except for the atomization rate (x_3). The range of this factor was narrowed to fall between 55 and 65 mm to optimize the production of fine particles suitable for pulmonary delivery. Among the responses, y_1 and y_3 were set to be minimized while y_2 was set to be maximized, and y_4 was chosen to be in the range of the experimentally obtained values. Equal weight (1) and importance (+++) were given to all

responses as they are integrated indicators of liposome stability after processing. Factor values associated with high desirability function which are fulfilling the maximum requirement of responses were selected and represented in **Table 11**. To validate the polynomial equations ability to predict the y_1, y_2, and y_3 responses (y_4 was not included due to poor model predictability), spray drying trials of liposomal ciprofloxacin dispersions using the optimum parameters was carried out for two protectants (i.e., sucrose and lactose). **Table 12** demonstrates the observed values and the predicted response range of the optimized formulations. These confirmatory runs using the optimized parameters produced CNL inhalation powders with characteristics very close to the predicted values, thus validating the predictive capability of the equations.

3.6. *Evaluation of optimized formulations for respiratory delivery and controlled drug release*

3.6.1. Moisture Content

Optimized sucrose and lactose formulations contained 2.5±0.3% w/w and 2.6±0.4% w/w residual moisture, respectively. Water acts as a plasticizer which can decrease the glass transition temperature (Tg) of the amorphous spray dried powder. Thus, residual moisture can affect the stability of powders upon storage as it may induce powder recrystallization and poor dispersion during inhalation (Chan et al., 2004; White et al., 2005). The TGA results indicated that optimized CNL formulations contained low moisture content of *ca* 3% w/w.

3.6.2. In vitro aerosol performance

The fine particle fraction (FPF) and emitted dose (ED) were 61.7% and 85.0%, respectively, for the optimized sucrose formulation. The lactose formulation showed improved aerosol performance with 76.2% FPF and 84.3% ED. The mass median aerodynamic diameter (MMAD) values were 2.5±0.3 and 1.8±0.3 µm for the sucrose and lactose formulations, respectively. Thus, these optimized CNL formulations showed superior aerosol performance suitable for inhalation delivery to the lungs.

3.6.3. In vitro assay of Cf release from liposomes

A controlled drug release was observed for both optimized formulations in comparison to the original liposomal Cf dispersion (control). Optimized sucrose and lactose formulations showed 90% drug release at 8 hours while the control had 90% released within 2 hours (**Fig. 8**). IVR assay results confirmed not only the preservation of the liposomal vesicles integrity

after spray drying, but also revealed the formation of nanocrystalline Cf structures which served the purpose of controlled drug release as reported previously (Barenholz, 2012; Cipolla et al., 2016c; Khatib et al., 2019; Khatib et al., 2020).

4. Conclusion

The method for generating CNL inhaled powders with optimal characteristics was determined using an experimental design methodology. By applying the BB design and response surface methodology, the spray drying parameters were modeled to produce CNL powders that were characterized for vesicle size, encapsulation efficiency, zeta potential and nanocrystal dimension. The results showed that the three protectants (sucrose, trehalose and lactose) have similarities in preserving the integrity of the liposomal vesicles during spray drying. However, trehalose was inferior compared to sucrose and lactose with respect to retention of the encapsulated drug. Although nanocrystals were generated regardless of the applied physiochemical parameters, stable liposomes and optimal encapsulation efficiency values were only achievable with the highest protectant amount. The most elongated nanocrystals were associated with the highest trehalose amount, but the degree of elongation was random for sucrose and lactose. The observed responses were close to the predicted values for the optimized nanocrystal generation method. Hence, mathematical equations can be used to predict the responses of liposome size, encapsulation efficiency and zeta potential from the method parameters applied. In conclusion, the Box–Behnken design of experiments is an economical way to extract the maximum amount of complex information for the CNL production method, with the process characterization and optimization achievable with a relatively small number of experimental runs in a short time.

Declaration of interest

This work is related to a provisional patent for the university of Sydney entitled with "Formation of ciprofloxacin nanocrystals within liposomal vesicles by spray drying for controlled drug release via inhalation" (CDIP Ref. Number IP [2019-024]) created by the originators Prof. Hak-Kim Chan and Ms. Isra Khatib.

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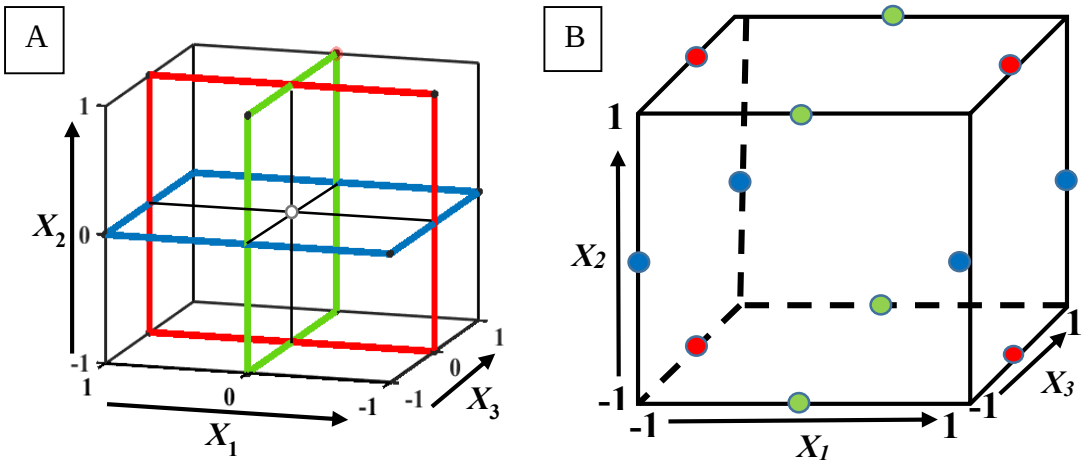

Fig. 1. Box–Behnken design (A) represented as three blocks; (B) as derived from a cube.

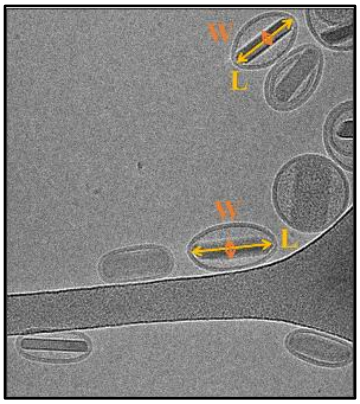

Fig. 2. Representation of the dimensions of ciprofloxacin nanocrystals inside liposomes.

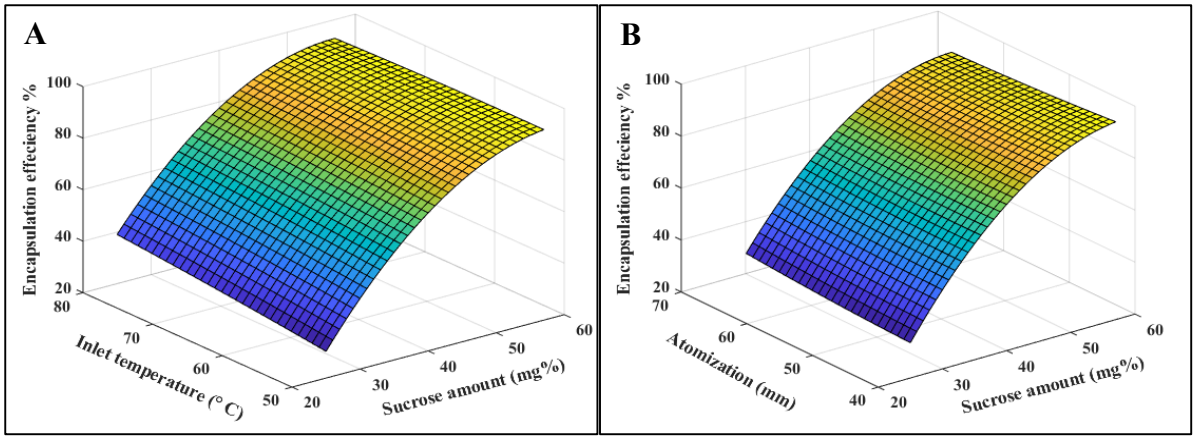

Fig. 3. Response surface plots showing the effect of sucrose amount (x_1) and drying temperature (x_2) [**A**] or atomization (x_3) [**B**] on drug EE%.

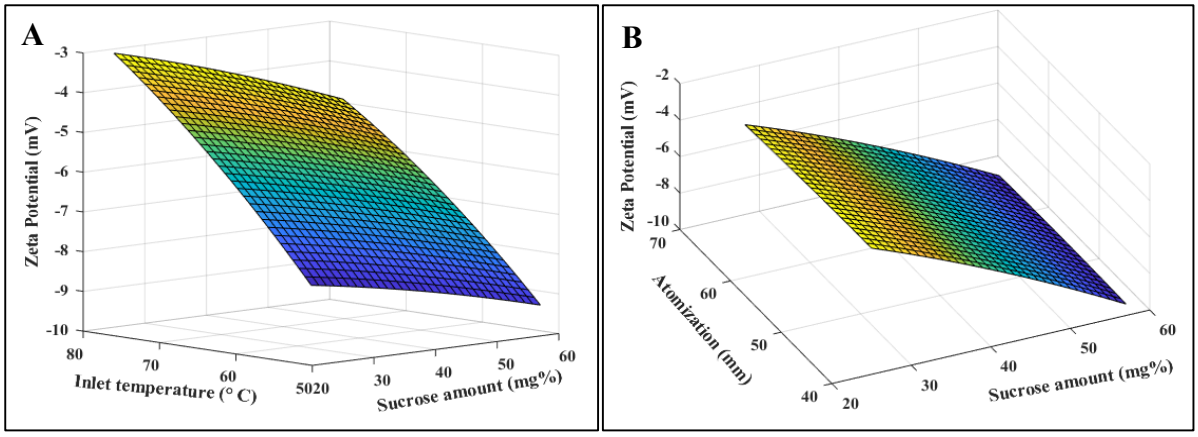

Fig. 4. Response surface plots showing the effect of sucrose amount (x_1) and drying temperature (x_2) [**A**] or atomization (x_3) [**B**] on zeta potential.

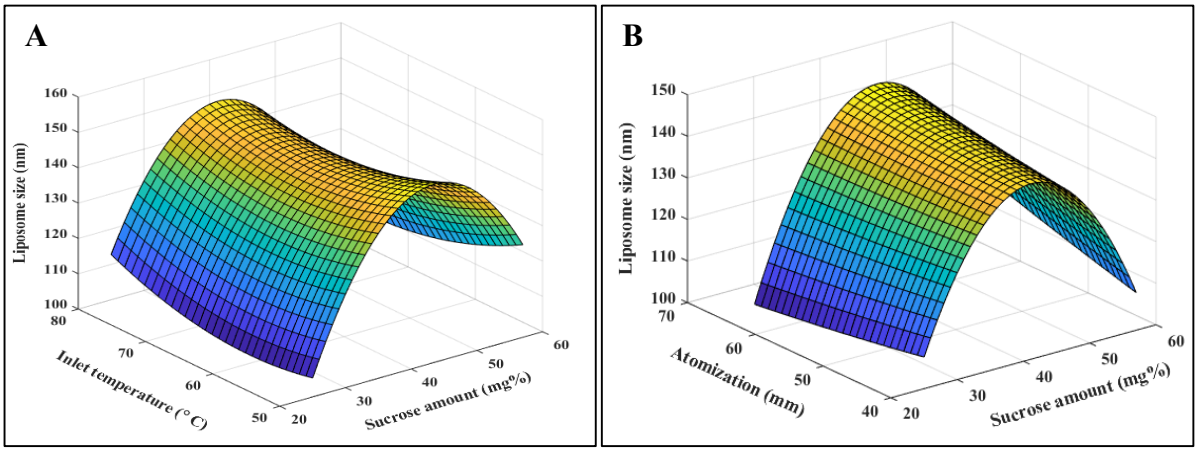

Fig. 5. Response surface plots showing the effect of sucrose amount (x_1) and drying temperature (x_2) [**A**] or atomization (x_3) [**B**] on liposome particle size.

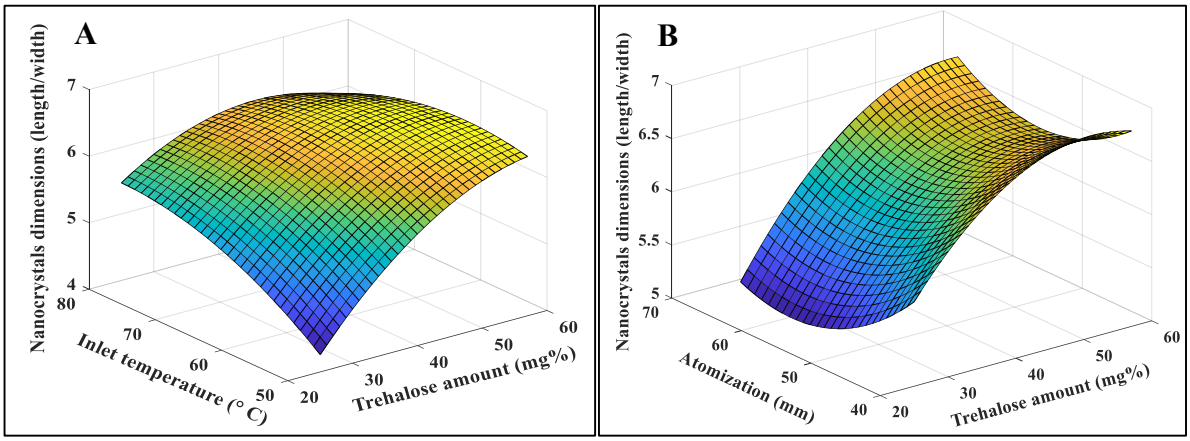

Fig. 6. Response surface plots showing the effect of trehalose amount (x_1) and drying temperature (x_2) [**A**] or atomization (x_3) [**B**] on nanocrystals dimensions.

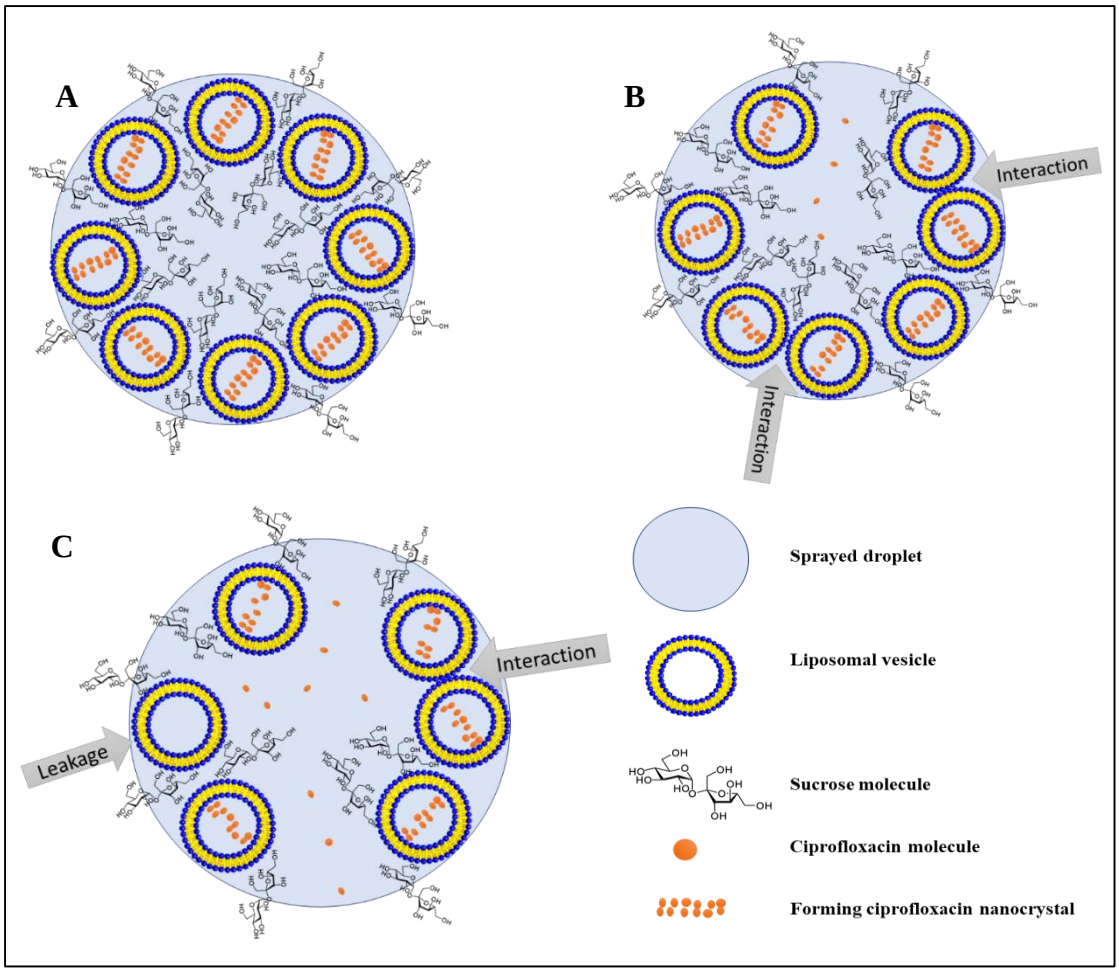

Fig. 7. Schematic representation of liposomal vesicles during drying with three levels of protectant: **A.** High, **B**. Medium, and **C**. Low.

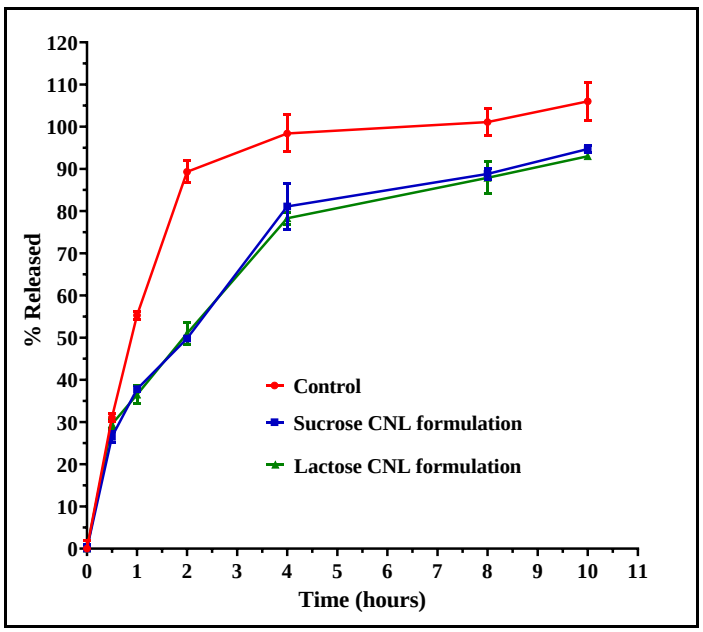

Fig. 8. *In vitro* release profiles of optimized CNL formulations containing sucrose or lactose as protectant in comparison to the control (original liposomal Cf dispersion). Mean with SD, n = 3.

Table 1. BB experimental design with coded values for factor levels of 15 experiments (BB1–BB15).

Experiment (run)	Factor and factor level		
	x_1	x_2	x_3
BB1	-1	-1	0
BB2	1	-1	0
BB3	-1	1	0
BB4	1	1	0
BB5	-1	0	-1
BB6	1	0	-1
BB7	-1	0	1
BB8	1	0	1
BB9	0	-1	-1
BB10	0	1	-1
BB11	0	-1	1
BB12	0	1	1
BB13	0	0	0
BB14	0	0	0
BB15	0	0	0

Table 2. Spray droplet size distribution measured by Spraytec laser diffraction at a liquid feed rate of 1.4 mL/min and different atomization gas flow rates. Mean ± SD, n=3.

Atomization gas flow (L/hr)	Rotameter height (mm)*	Droplet median volume (µm)	Span	Comments
819	65	9.2±0.30	1.46±0.15	Maximum atomization can be set on spray dryer
742	60	16.3±0.3	1.36±0.04	
670	55	18.3±0.0	1.35±0.01	
601	50	20.0±0.2	1.17± 0.00	
536	45	22.7±0.0	1.17± 0.00	
473	40	26.0±0.3	1.21± 0.00	Minimum atomization to achieve efficient drying

* The rotameter is an indicator of the spray gas flow. The table gives a linkage between the indicated height and actual gas throughput.

Table 3. Factors and factor levels investigated in the BB experimental design.

Factor		Unit	Factor level		
			-1	0	1
x_1	Sugar amount	%w/w	25	41	57
x_2	Inlet temperature	°C	50	65	80
x_3	Atomization (gas flow) or rotameter height	L/hr mm	473 40	648 53	819 65

Table 4. Responses selected and the constraints used in the BB experimental design.

Response	Method	Constraints	Rationale
y_1: Liposome size	Nanosizer (Malvern) dynamic light scattering (intensity)	Size values close to 100 nm (original liposome size) (Cipolla et al., 2016c; Khanal et al., 2020)	Liposome size is an indicator of liposomal stability after processing
y_2: Drug EE	Nanosep device 10 K Omega and HPLC	EE% close to 99.9% (original liposome EE) (Cipolla et al., 2016c; Khanal et al., 2020)	EE is an indicator of drug entrapment efficiency after processing
y_3: Zeta Potential	Nanosizer (Malvern) dynamic light scattering	Zeta value close to -10.0 mV (zeta potential of original liposomes) [a]	Surface charge is an indicator of the repulsive forces between liposomal particles
y_4: Dimensions of ciprofloxacin nanocrystals (length/width) (L/W)	Cryogenic transmission electron microscopy and ImageJ software	No constraints [b]	Effect of process parameters on the dimensions of the generated nanocrystals

[a] Reported zeta potential value of liposomes composed of hydrogenated soy phosphatidylcholine (HSPC) (Chen et al., 2012).
[b] Optimum values are unavailable.

Table 5. Box–Behnken design with the results for sucrose as the protectant (Experimental runs with the highest EE values are shown in bold.)

Run No.	Actual variables values			Responses and results			
	x_1	x_2	x_3	Size (nm) (RSD%)	EE% (SD)	Zeta Potential (mV) (SD)	Nanocrystals dimensions (L/W) (SD)
1	25	50	53	103.6 (3.9)	30.1 (2.6)	- 3.31 (0.25)	5.89 (3.25)
2	57	50	53	124.7 (2.0)	93.1 (0.3)	- 8.75 (0.37)	6.88 (2.44)
3	25	80	53	114.3 (0.5)	39.9 (1.0)	-3.58 (0.61)	4.27 (2.33)
4	**57**	**80**	**53**	**116.5 (1.1)**	**93.0 (0.2)**	**- 9.68 (0.75)**	**4.05 (2.00)**
5	25	65	40	110.4 (3.6)	32.2 (1.2)	- 3.05 (0.13)	4.87 (2.67)
6	**57**	**65**	**40**	**111.6 (1.6)**	**95.3 (0.4)**	**- 9.72 (0.75)**	**6.34 (3.36)**
7	25	65	65	98.5 (0.5)	38.5 (0.9)	- 3.02 (0.28)	4.29 (2.34)
8	**57**	**65**	**65**	**119.5 (1.0)**	**93.7 (0.2)**	**- 9.42 (0.34)**	**6.33 (3.4)**
9	41	50	40	144.8 (4.1)	78.4 (2.4)	- 6.05 (0.24)	5.94 (2.6)
10	41	80	40	144.6 (2.4)	79.6 (0.2)	- 5.74 (0.47)	5.34 (2.52)
11	41	50	65	158.5 (3.2)	74.9 (0.7)	- 6.74 (0.85)	5.82 (2.76)
12	41	80	65	148.2 (2.0)	79.0 (0.4)	- 5.99 (0.43)	7.63 (3.96)
13	41	65	53	143.3 (3.7)	75.1 (0.3)	- 5.96 (0.73)	5.28(2.59)
14	41	65	53	142.0 (1.8)	78.3 (0.9)	- 5.81 (0.42)	5.44 (2.19)
15	41	65	53	145.2 (1.2)	80.2 (1.5)	- 6.14 (0.47)	5.54 (2.75)

Table 6. Box–Behnken design with the results for trehalose as the protectant
(Experimental runs with the highest EE values are shown in bold.)

Run No.	Actual variables values			Responses and results			
	x_1	x_2	x_3	Size (nm) (RSD%)	EE% (SD)	Zeta Potential (mV) (SD)	Nanocrystals dimensions (L/W) (SD)
1	25	50	53	125.7 (2.9)	35.0 (2.66)	-5.74 (0.42)	4.40 (2.04)
2	57	50	53	122.6 (2.1)	86.0 (3.70)	-10.63 (0.58)	6.48 (3.04)
3	25	80	53	121.3 (3.6)	34.2 (4.72)	-4.91 (0.38)	5.38 (2.7)
4	**57**	**80**	**53**	**112.0 (0.1)**	**89.7 (2.15)**	**-12.48 (1.59)**	**5.81 (3.16)**
5	25	65	40	110.9 (5.2)	32.0 (1.59)	-4.48 (0.32)	6.05 (3.51)
6	**57**	**65**	**40**	**109.0 (1.7)**	**89.5 (3.01)**	**-9.26 (0.38)**	**7.15 (3.37)**
7	25	65	65	129.3 (3.2)	34.0 (3.78)	-4.00 (0.08)	4.94 (2.70)
8	57	65	65	116.4 (1.6)	87.6 (1.55)	-9.87 (0.32)	6.57 (2.87)
9	41	50	40	141.2 (4.2)	68.5 (1.75)	-6.61 (0.66)	5.82 (3.26)
10	41	80	40	139.5 (2.3)	59.6 (1.72)	-5.89 (1.06)	6.50 (3.38)
11	41	50	65	146.6 (2.5)	67.7 (2.57)	-6.25 (0.39)	6.18 (3.45)
12	41	80	65	137.2 (3.5)	68.1 (3.01)	-6.32 (0.67)	6.72 (3.00)
13	41	65	53	141.4 (3.1)	63.7 (2.09)	-6.36 (0.67)	5.60 (2.73)
14	41	65	53	140.1 (2.3)	69.2 (2.66)	-6.45 (0.87)	7.00 (3.42)
15	41	65	53	141.1 (2.0)	66.5 (0.78)	-7.33 (0.19)	6.08 (3.27)

Table 7. Box–Behnken design with the results for lactose as the protectant
(Experimental runs with the highest EE values are shown in bold.)

Run No.	Actual variables values			Responses and results			
	x_1	x_2	x_3	Size (nm) (RSD%)	EE% (SD)	Zeta Potential (mV) (SD)	Nanocrystals dimensions (L/W) (SD)
1	25	50	53	114.2 (1.2)	41.1 (1.0)	-3.95 (0.55)	4.48 (1.99)
2	57	50	53	140.3 (4.1)	91.0 (0.8)	-7.30 (0.80)	5.65 (2.49)
3	25	80	53	123.3 (4.3)	37.0 (0.9)	-4.77 (0.64)	4.92 (2.50)
4	**57**	**80**	**53**	**120.6 (4.9)**	**94.6 (0.6)**	**-8.84 (0.93)**	**6.31 (2.80)**
5	25	65	40	120.5 (4.3)	35.0 (1.8)	-3.79 (0.21)	5.12 (2.49)
6	**57**	**65**	**40**	**123.6 (2.7)**	**95.3 (0.5)**	**-7.81 (0.86)**	**5.22 (2.76)**
7	25	65	65	125.0 (3.6)	43.1 (3.0)	-4.08 (0.27)	4.51 (2.22)
8	**57**	**65**	**65**	**115.4 (2.1)**	**94.4 (0.6)**	**-9.32 (0.73)**	**5.43 (2.70)**
9	41	50	40	148.8 (3.6)	69.1 (2.8)	-6.11 (0.38)	5.82 (3.26)
10	41	80	40	138.0 (2.4)	72.0 (4.2)	-5.61 (0.37)	5.43 (2.46)
11	41	50	65	153.6 (3.8)	76.2 (1.0)	-5.98 (0.43)	5.72 (2.70)
12	41	80	65	143.4 (0.7)	80.3 (2.2)	-6.49 (0.92)	7.80 (3.68)
13	41	65	53	137.0 (1.1)	79.4 (2.7)	-6.47 (0.37)	6.84 (3.04)
14	41	65	53	145.1 (4.8)	73.7 (2.2)	-5.48 (0.41)	5.19 (2.35)
15	41	65	53	139.3 (3.0)	78.4 (0.3)	-6.54 (0.90)	6.11(2.50)

Table 8. Actual equations of the four responses for the three protectants (sucrose, trehalose and lactose).

Protectant	Response	Model Equation	No.
Sucrose	y_1: Particle size	$-38.38 + 11.22x_1 - 1.55x_2 - 0.24x_3 - 0.13x_1^2 + 0.023x_2^2 + 0.0022x_3^2 - 0.0197x_1x_2 + 0.024x_1x_3 - 0.013x_2x_3$	**Eq. 3**
	y_2: Cf EE	$-128.30 + 7.31x_1 + 0.58x_2 - 0.16x_3 - 0.053x_1^2 - 0.0017x_2^2 + 0.003x_3^2 - 0.0103x_1x_2 - 0.0095x_1x_3 + 0.0037x_2x_3$	**Eq. 4**
	y_3: Zeta potential	$-1.49 - 0.079x_1 + 0.051x_2 - 0.014x_3 - 0.00104x_1^2 - 0.00042x_2^2 - 0.00039x_3^2 - 0.00069x_1x_2 - 0.00032x_1x_3 + 0.00056x_2x_3$	**Eq. 5**
	y_4: Nanocrystal dimension	$24.20 + 0.22x_1 - 0.31x_2 - 0.51x_3 - 0.0017x_1^2 + 0.0013x_2^2 + 0.0028x_3^2 - 0.0013x_1x_2 + 0.00069x_1x_3 + 0.0031x_2x_3$	**Eq. 6**
Trehalose	y_1: Particle size	$-70.45 + 8.15x_1\ \ 0.66x_2 + 2.64x_3 - 0.088x_1^2 + 0.0095x_2^2 - 0.0111x_3^2 - 0.0065x_1x_2 - 0.0132x_1x_3 - 0.0099x_2x_3$	**Eq. 7**
	y_2: Cf EE	$-3.29 + 3.32x_1 - 0.86x_2 - 0.21x_3 - 0.0204x_1^2 - 9.26e\text{-}05x_2^2 - 0.0028x_3^2 + 0.0047x_1x_2 - 0.0047x_1x_3 + 0.0119x_2x_3$	**Eq. 8**
	y_3: Zeta potential	$-14.30 + 0.45x_1 + 0.48x_2 - 0.50x_3 - 0.0046x_1^2 - 0.0024x_2^2 + 0.0059x_3^2 - 0.0028x_1x_2 - 0.0013x_1x_3 - 0.0010x_2x_3$	**Eq. 9**
	y_4: Nanocrystal dimension	$-1.60 + 0.25x_1 + 0.26x_2 - 0.26x_3 - 0.0016x_1^2 - 0.0013x_2^2 + 0.0022x_3^2 - 0.0017x_1x_2 + 0.00064x_1x_3 - 0.00018x_2x_3$	**Eq. 10**
Lactose	y_1: Particle size	$5.59 + 9.41x_1 - 1.66x_2 + 0.0085x_3 - 0.0795x_1^2 + 0.0199x_2^2 + 0.0059x_3^2 - 0.030x_1x_2 - 0.0153x_1x_3 + 0.00077x_2x_3$	**Eq. 11**
	y_2: Cf EE	$-116.32 + 4.76x_1 + 0.74x_2 + 1.11x_3 - 0.0365x_1^2 - 0.0084x_2^2 - 0.0052x_3^2 + 0.0080x_1x_2 - 0.0108x_1x_3 + 0.0015x_2x_3$	**Eq. 12**
	y_3: Zeta potential	$-6.63 + 0.037x_1 + 0.036x_2 + 0.094x_3 - 0.000496x_1^2 + 0.00034x_2^2 + 0.00024x_3^2 - 0.00075x_1x_2 - 0.0015x_1x_3 - 0.0013x_2x_3$	**Eq. 13**
	y_4: Nanocrystal dimension	$13.0 + 0.25x_1 - 0.27x_2 - 0.19x_3 - 0.0036x_1^2 + 0.00092x_2^2 - 0.00037x_3^2 + 0.00023x_1x_2 + 0.00099x_1x_3 + 0.0032x_2x_3$	**Eq. 14**

Table 9. Analysis of variance for all four responses y_1, y_2, y_3 and y_4.

Protectant	Response	Model Statistics		Coefficient of determination	
		F value	*p*-value	R^2	Adjusted R^2
Sucrose	y_1	**32.82**	**0.0006**	0.9830	0.9530
	y_2	**164.6**	**< 0.0001**	0.9966	0.9906
	y_3	**35.51**	**0.0005**	0.9850	0.9570
	y_4	0.6972	0.6997	0.5565	0.0000
Trehalose	y_1	**18.62**	**0.0030**	0.9710	0.9190
	y_2	**84.20**	**< 0.0001**	0.9934	0.9816
	y_3	**34.03**	**0.0006**	0.9840	0.9550
	y_4	1.950	0.2397	0.7780	0.3783
Lactose	y_1	**6.180**	**0.0300**	0.9180	0.7690
	y_2	**99.85**	**< 0.0001**	0.9945	0.9845
	y_3	**10.57**	**0.0090**	0.9500	0.8600
	y_4	1.730	0.2837	0.7567	0.3188

Table 10. Coded factor effects and associated *p*-values for all four responses (Significant effect (p<0.05) of factors on individual responses are shown in bold).

Protectant type	Response		Factors									
			Intercept	x_1	x_2	x_3	x_1x_2	x_1x_3	x_2x_3	x_1^2	x_2^2	x_3^2
Sucrose	y_1	*b* (0-9)	143	**5.59**	-0.95	1.66	-4.73	4.95	-2.46	**-33.88**	5.15	0.44
		p-value		**0.012**	0.54	0.3	0.07	0.061	0.28	**< 0.0001**	0.061	0.84
	y_2	*b* (0-9)	77.86	**29.34**	1.86	0.075	-2.48	-1.98	0.75	**-13.45**	-0.4	0.51
		p-value		**< 0.0001**	0.068	0.93	0.081	0.14	0.54	**< 0.0001**	0.75	0.69
	y_3	*b* (0-9)	-5.97	**-3.0778**	-0.019	-0.076	-0.17	0.075	0.099	-0.27	-0.094	-0.07
		p-value		**< 0.0001**	0.91	0.68	0.53	0.77	0.7	0.34	0.73	0.8
	y_4	*b* (0-9)	5.41	0.53	-0.42	0.2	-0.3	0.13	0.57	-0.44	0.29	0.48
		p-value		0.23	0.33	0.63	0.61	0.82	0.35	0.48	0.64	0.44
Trehalose	y_1	*b* (0-9)	141	-3.35	-3.22	**3.61**	-1.55	-2.74	-1.94	**-22.6**	2.13	-1.73
		p-value		0.05	0.056	**0.039**	0.44	0.2	0.34	**< 0.0001**	0.32	0.41
	y_2	*b* (0-9)	66.43	**27.22**	-0.75	0.98	1.13	-1	2.38	**-5.22**	-0.021	-0.43
		p-value		**< 0.0001**	0.49	0.37	0.46	0.51	0.15	**0.016**	0.99	0.78
	y_3	*b* (0-9)	-6.71	**-2.88**	-0.042	-0.025	**-0.67**	-0.28	-0.21	**-1.18**	-0.55	**0.99**
		p-value		**< 0.0001**	0.82	0.9	**0.047**	0.32	0.46	**0.0067**	0.095	**0.014**
	y_4	*b* (0-9)	6.23	**0.65**	0.19	-0.14	-0.41	0.13	-0.04	-0.42	-0.29	0.36
		p-value		**0.027**	0.4	0.54	0.22	0.68	0.9	0.23	0.39	0.29
Lactose	y_1	*b* (0-9)	140	2.17	-3.95	0.81	-7.20	-3.02	0.20	**-20.35**	4.48	1.04
		p-value		0.36	0.13	0.72	0.066	0.37	0.95	**0.0014**	0.22	0.76
	y_2	*b* (0-9)	77.05	**27.43**	0.81	**2.83**	1.93	-2.27	0.26	**-9.35**	-1.90	-0.76
		p-value		**< 0.0001**	0.43	**0.031**	0.21	0.15	0.85	**0.0011**	0.23	0.61
	y_3	*b* (0-9)	-6.15	**-2.08**	-0.29	-0.32	-0.18	-0.3	-0.26	-0.18	0.075	0.028
		p-value		**0.0002**	0.25	0.21	0.59	0.39	0.44	0.71	0.83	0.94
	y_4	*b* (0-9)	6.04	0.44	0.34	0.23	0.055	0.21	0.61	-0.91	0.21	-0.05
		p-value		0.14	0.24	0.4	0.88	0.58	0.15	0.059	0.6	0.89

Table 11. Optimal parameters for the three protectants and desirability values**.**

Protectant type	Parameters			Desirability
	Protectant amount (x_1): %w/w	Inlet temperature (x_2): °C	Atomization (x_3): L/hr	
Sucrose	57	80	742	0.894
Trehalose	57	80	742	0.973
Lactose	57	65	742	0.921

Table 12. Predicted range and observed experimental values of optimized formulations.

Protectant	**Response**	**Predicted range (95%PI)**	**Observed**
Sucrose	y_1: Liposomes size (nm)	105.5 – 129.0	125.0 ± 1.0
	y_2: Drug EE (%w/w)	85.7 – 98.7	90.2 ± 1.6
	y_3: Zeta Potential (mV)	-10.9 – -8.2	-10.0 ± 1.1
Lactose	y_1: Liposomes size (nm)	107.3 – 135.3	124.4 ± 5.5
	y_2: Drug EE (%w/w)	89.1 – 101.3	89.2 ± 1.1
	y_3: Zeta Potential (mV)	-10.1 – -7.3	-9.9 ± 1.0

*Conflict of Interest

Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

This work is related to a provisional patent for the university of Sydney entitled with “Formation of ciprofloxacin nanocrystals within liposomal vesicles by spray drying for controlled drug release via inhalation” (CDIP Ref. Number IP [2019-024]) created by the originators Prof. Hak-Kim Chan and Ms. Isra Khatib.

Authorship Statement

Manuscript title: Modeling of a spray drying method to produce ciprofloxacin nanocrystals inside the liposomes utilizing a response surface methodology: Box-Behnken experimental design

Authorship contributions

Isra Khatib: Conceptualization, Data Curation, Formal Analysis, Investigation, Software, Methodology, Writing - Original Draft.

Michael Y.T. Chow: Formal Analysis, Software, Writing - Review & Editing.

Juanfang Ruan: Investigation, Data Curation, Writing - Review & Editing.

David Cipolla: Conceptualization, Resources, Writing - Review & Editing.

Kim Chan: Conceptualization, Funding Acquisition, Resources, Project Administration, Supervision, Writing - Review & Editing.